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Type III Secretion of the *Salmonella* Effector Protein SopE Is Mediated via an N-Terminal Amino Acid Signal and Not an mRNA Sequence

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Type III secretion systems (TTSS) are virulence-associated components of many gram-negative bacteria that translocate bacterial proteins directly from the bacterial cytoplasm into the host cell. The *Salmonella* translocated effector protein SopE has no consensus cleavable amino-terminal secretion sequence, and the mechanism leading to its secretion through the *Salmonella* pathogenicity island 1 (SPI-1) TTSS is still not fully understood. There is evidence from other bacteria which suggests that the TTSS signal may reside within the 5′ untranslated region (UTR) of the mRNA of secreted effectors. We investigated the role of the 5′ UTR in the SPI-1 TTSS-mediated secretion of SopE using promoter fusions and obtained data indicating that the mRNA sequence is not involved in the secretion process. To clarify the proteinaceous versus RNA nature of the signal, we constructed frameshift mutations in the amino-terminal region of SopE of *Salmonella enterica* serovar Typhimurium SL1344. Only constructs with the native amino acid sequence were secreted, highlighting the importance of the amino acid sequence versus the mRNA sequence for secretion. Additionally, we obtained frameshift mutation data suggesting that the first 15 amino acids are important for secretion of SopE independent of the presence of the chaperone binding site. These data shed light on the nature of the signal for SopE secretion and highlight the importance of the amino-terminal amino acids for correct targeting and secretion of SopE via the SPI-1-encoded TTSS during host cell invasion.

Gram-negative pathogens employ complex syringe-like macromolecular structures termed type III secretion systems (TTSS) to facilitate translocation of effector proteins into the cytoplasm of host cells (22–24). Two distinct TTSS encoded in *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2 are present in *Salmonella* (22). The TTSS encoded by SPI-2 (TTSS-2) is important for intracellular survival and replication of the pathogen (9, 18, 19). The TTSS encoded by SPI-1 (TTSS-1) mediates invasion of nonphagocytic epithelial cells located on the intestinal mucosa (14). Invasion of enterocytes and M cells results in villus blunting and loss of absorptive surfaces, a characteristic pathology of enteritis (41), and mutations in TTSS-1 greatly reduce enteropathogenicity in calves (1, 39, 42). SPI-1 encodes several TTSS-1-secreted effector proteins and their chaperones. In addition, TTSS-1 can translocate a number of effector proteins encoded by genes located elsewhere on the chromosome (44).

The following three effector proteins have been shown to have paramount importance for enteropathogenicity: SopE, SopE2, and SopB (21, 45). The effector protein SopE is a guanine nucleotide exchange factor that interacts with Cdc42, promoting actin cytoskeletal reorganization and mediating internalization in the host cell (16, 17).

Unlike proteins excreted by the common secretory bacterial systems, TTSS effector proteins are not proteolytically cleaved, and they do not appear to possess conserved amino acid sequence motifs in the regions involved in secretion and translocation (22). So far, the exact mechanism by which TTSS specifically transport effectors like SopE and SopE2 without any obvious recognition signal remains a mystery. Existing data for the translocated Yop proteins of *Yersinia* suggest the existence of two independent signals present in the amino terminus of the protein that direct secretion and translocation (37). In *Yersinia*, TTSS secretion is facilitated by the N-terminal mRNA sequence corresponding to the first 15 codons (3, 37), while translocation is dependent on the region between amino acids 50 and 100 (6, 36, 37). Both secretion and translocation of these effectors are dependent on chaperones that bind these important regions (43). Certain TTSS substrates are involved in the regulation of effector protein synthesis or secretion by mechanisms that involve binding to the 5′ untranslated region (UTR) region of the effector protein or other amino-terminal regions (2, 7).

In *Salmonella*, a conserved amino acid sequence containing leucine-rich repeats has been shown to direct intracellular type III secretion of effector proteins encoded outside SPI-1 and SPI-2 (28, 40). However, no such signal has been clearly found to mediate secretion of effector proteins like SopE and SopE2. It has been shown that the region between residues 15 and 78 of SopE is responsible for binding the chaperone InvB (25). The chaperone binding segment was also shown to confer greater stability to SopE but not to SopE2 (12).

We investigated the amino-terminal region of SopE using frameshift mutations in order to clarify the roles of the mRNA and the 5′ UTR in the secretion of SopE. Our data suggest that secretion of SopE occurs independently of the 5′ UTR and

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TABLE 1. Strains used in this study

Strain	Genotype	Reference
SL1344	Wild type	20
SL3261	SL1344 *aroA*	20
RM69	Δ*spi-1*::*kan*	29
SL3262	SL3261 Δ*spi-1*::*kan*	This study

that, in contrast to the *Yersinia* system, mRNA is not likely to contribute to the targeting and secretion process. We also determined the importance of the first 15 codons in the secretion process and investigated the significance of putative motifs present in the amino-terminal region of *sopE* mRNA.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Salmonella enterica* serovar Typhimurium strains were grown in Luria-Bertani (LB) medium at 37°C and 200 rpm unless indicated otherwise. Antibiotics were added to cultures at designated concentrations (35). Each of the strains described here displayed growth rates identical to those of its parental strain. Table 1 shows the strains used. Electrocompetent cells were prepared by the standard lab protocol. Briefly, 25 ml of fresh LB medium was inoculated (1:25) with an overnight culture of a strain in LB medium and grown at 37°C to an optical density at 600 nm of 0.4. The cells were then washed on ice with 1 and 0.5 volumes of double-distilled H_2O, followed by 0.1 volume of 10% glycerol. The cells were finally resuspended in 0.01 volume of 10% glycerol, divided into aliquots, and stored at −80°C. DNA to be electroporated (100 μg) was mixed with cells and subjected to 2.5 kV in an electroporator (Bio-Rad). Cells were recovered in 750 μl of LB medium containing 20 mM glucose at 37°C for 90 min and plated on selective LB agar plates.

Plasmid construction. All recombinant DNA techniques used were based on protocols described by Sambrook et al. (35). The PCR primers used (Sigma-Genosys Ltd.) are listed in Table 2. PCRs were carried out by using Taq2000 DNA polymerase (Stratagene), and ligation was performed by using a Quick Ligation kit (New England Biolabs). Sequencing was carried out by using an ABI sequencer (Molecular Biology Unit, University of Newcastle). The sequence numbers below correspond to the numbers in the previously published *S. enterica* serovar Typhimurium LT2 sequence (26).

To determine the role of the 5′ UTR, a fragment containing just the *sopE* coding sequence was amplified with primers OMW39 and OMW40 and cloned into EcoRI-HindIII-digested pBAD24 (Table 3) to generate $pMKP_{BAD}$, which expressed *sopE* with an amino-terminal 8-amino-acid Strep tag fusion under control of the inducible arabinose promoter (P_{BAD}). Additionally, the promoter region of *sopE* was amplified with primers MHK05 (BamHI) and MHK06a (KpnI) and cloned upstream of a promoterless β-lactamase gene on BamHI-KpnI-digested pAJR104 (Table 3), giving rise to $pMKP_{sopE}$.

To determine the minimum region necessary for SopE secretion, primers OJH62 and OJH65 were used to amplify the full *sopE* coding region, including P_{sopE} and the 5′ UTR. The resulting product, which comprised *sopE* fused to a region encoding an 11-amino-acid herpes simplex virus (HSV) epitope tag at the 3′ end of *sopE*, was cloned into a transcriptionally silent region of pBR322, giving pMK240 (Fig. 1). This plasmid was also used as a template in inverse PCRs performed with OJH62 in combination with OJH63 to create pMK20. Furthermore, anchor primer OMW33 was used in inverse PCRs in combination with primers OMW36, OMW35, and OMW49 to generate pMK40, pMK60, and pMK70, respectively.

Altered frame vectors pMKAF70, pMKAF15, and pMK70Δ were constructed in two steps. Initially, a single addition initiating a frame change (adding G at position 4481803), as well as an EcoRV restriction site (GATATC at positions 4481794 to 4481799) before the start codon and a thymine substitution at codon 12 (T→A at position 4481835), were engineered in plasmid pMK70 inverse PCR using primers MHK22 and MHK23, resulting in pMKAF70* (Table 3). Subsequently, thymine substitutions at codon 57 (T→C at position 4481970) and codon 60 (T→G at position 4481979) were introduced to remove stop codons via inverse PCRs performed with primers MK04 and OMW33, resulting in pMKAF70 (Table 3). Primers MHK22 and MHK23a were used to introduce two frame alterations in pMK70 that altered the frame between codon 1 (addition of G at position 4481803) and codon 15 (deletion of A at position 4481847), resulting in pMKAF15. Primers MHK13a and MHK14a were used in inverse PCRs in which pMK70 was used to induce a frame alteration between codon 50 (deletion of T at position 4481950) and codon 57 (addition of A at position 4481979), as well as an EcoRV restriction site (GATATC at positions 4481961 to 4481966) and a thymine substitution at codon 54 (T→G at position 4481960), resulting in pMK70Δ. All constructs were sequenced on both the sense and antisense strands by using primers OMW57 and OMW58, respectively, confirming the DNA sequence.

RNA techniques. RNA isolation was carried out by using an SV Total RNA isolation system (Promega) according to the manufacturer's instructions. RNA was stored at −80°C. Northern dot blotting was performed on nylon membranes that were positively charged (Roche) by using 10 μg of RNA per dot and a Dig Northern blot starter kit (Roche) according to the manufacturer's instructions. To measure mRNA from the fusion constructs, digoxigenin-labeled OMW33 (DIG oligonucleotide tailing kit, second generation; Roche) was used to prime specifically for HSV-tag-encoding mRNA. The background was measured in an isogenic strain not expressing the HSV tag. Densitometry was carried out by using a Gel Doc 1000 system (Bio-Rad).

TABLE 2. Primers used in this study

Primer	Site	Sequence (5′-3′)[a]
MHK04	SpeI	CGCACTAGTCCTCGGTGAAAGTGTGTTGCAGAAGACTCCGTGTTCTTGTGCGAAATAAAACGTTCCG
MHK13a	EcoRV	CGCGATATCCAACGTTCCGTAATTTTGACCTTAATTCGATGAAGTG
MHK14a	EcoRV	CGGAACGTTGGATATCGCATAAGAAACACTGAGTCTTCTGCAACACACTTTC
MHK22	EcoRV	GCCGATATCATCCTTTTATATGTACATAATTC
MHK23	EcoRV	CCGGATATCCGTGGACAAAAATAACTTTATCTCCCCAGAATTTAAGAATCCAAAAACAGGAAACC
MHK23a	EcoRV	CCGGATATCCGTGGACAAAAATAACTTTATCTCCCCAGAATTTAAGAATCCAAAACAGGAAACC
MHK05	BamHI	CGCGGATCCTGAGCGAAAAG
MHK06a	KpnI	GCGGGTACCACGGTAATGATCCTTTTATATG
OMW33	SpeI	CGCACTAGT**CAGCCGGAACTGGCGCCGGAAGATCCGGAAGAT**
OMW35	SpeI	CGCACTAGTCTCAGTGTTCTTATGCGAAATA
OMW36	SpeI	CGCACTAGTATTTTTTACTGCGAGAATACTTTTTG
OMW39	EcoRI	CGCGAATTCGTGACAAAAATAACTTTATCTC
OMW40	HindIII	CGCAAGCTTTTATTTTTCGAACTGCGGGTGGCTCCAGGGAGTGTTTTGTATATATTTA
OMW49	SpeI	CGCACTAGTCGTTTGAAGCATAAAATCTTTA
OMW57		TCATGTTTGACAGCTTATCATCGA
OMW58		GTGATGCCGGCCACGATGCGTCCG
OJH62	HindIII	CCCAAGCTTTAATTCATCAATCAGATGGAC
OJH63	BamHI	CGCGGATCC**ATCTTCCGGATCTTCCGGCGCCAGTTCCGGCTG**TAGTGTGGTTTCCTGTTTTTGGATTC
OJH65	BamHI	CGCGGATCC**ATCTTCCGGATCTTCCGGCGCCAGTTCCGGCTG**GGGAGTGTTTTGTATATATTTATTAGC

[a] Restriction sites are underlined; sequences encoding the HSV tag are indicated by boldface type; and a sequence encoding a Strep tag is indicated by dashed underlining.

TABLE 3. Plasmids used in this study

Plasmid	Description[a]	Reference
pBR322	Cloning vector (signal-containing *bla*)	38
pACYC184	Cloning vector	10
pAJR104	pACYC184 (signalless *bla*)	A. J. Roe (unpublished data)
pBAD24	Expression vector	15
pMKP$_{BAD}$	pBAD24tag with *sopE* under P$_{BAD}$ control	This study
pMKP*sopE*	pAJR104 with *bla* under P$_{sopE}$ control	This study
pMK240	pBR322 (P$_{sopE}$, expressing SopE240-HSV tag)	This study
pMK70	pBR322 (P$_{sopE}$, expressing SopE70-HSV tag)	This study
pMK60	pBR322 (P$_{sopE}$, expressing SopE60-HSV tag)	This study
pMK40	pBR322 (P$_{sopE}$, expressing SopE40-HSV tag)	This study
pMK20	pBR322 (P$_{sopE}$, expressing SopE20-HSV tag)	This study
pMKAF70*	pMK70 (G added at position 4481803 and T→A at position 4481835)	This study
pMKAF70	pAF70* (T→C at position 4481970 and T→G at position 4481979)	This study
pMKAF15	pAF70* (A deleted at position 4481847)	This study
PMK70Δ	pMK70 (T deleted at position 4481950, T→G at position 4481960, A added at position 4481979, and GATATC at positions 4481961 to 4481966)	This study

[a] The nucleotide positions are the positions in the *sopE* sequence of the previously published *S. enterica* serovar Typhimurium LT2 genome sequence (26).

Protein techniques and in silico informatics. *Salmonella* strains harboring various expression constructs were grown in LB medium supplemented with 0.3 M NaCl under conditions that stimulated expression of the SPI-1 TTSS (13). Whole cells and culture supernatants were separated by centrifugation at 13,000 × *g* for 10 min. Whole cells were resuspended in an appropriate volume of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer. Culture supernatants were filter sterilized (pore size, 0.22 μm), and proteins were precipitated with ammonium sulfate (4 g/10 ml of supernatant) overnight at 4°C. Precipitated secreted proteins and pelleted cells were resuspended in phosphate-buffered saline and then combined with an equal volume of sample buffer (Bio-Rad). Whole-cell and culture supernatant samples were electrophoresed on an SDS—12% PAGE gel and transferred on Protran nitrocellulose transfer membranes (Schleicher & Schuell) by using a wet transfer apparatus (Bio-Rad). A Western blot analysis was performed by using a mouse HSV-tagged antibody (Novagen) or a mouse β-lactamase monoclonal antibody (QED Bioscience Inc.) coupled with a goat anti-mouse horseradish peroxidase-labeled secondary antibody (DakoCytomation). Detection was carried out by using 4-chloro-1-naphthol (Sigma) according to the manufacturer's instructions. Protein sequence comparisons and alignment were conducted by using the GeneJockey II software package (Biosoft), and motif searches were carried out by using Hits (http://hits.isb-sib.ch/) (30).

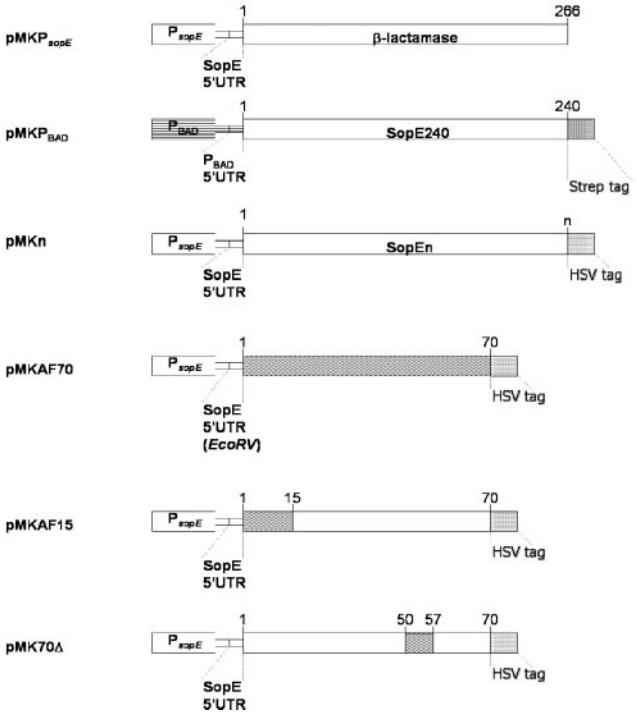

FIG. 1. Schematic diagram of SopE constructs used in this study. Regions with horizontal lines represent foreign promoter and 5′ UTR sequences. Stippled regions represent tag-encoding sequences (Strep or HSV). Truncation constructs are indicated by their sizes, as denoted by n (n = 20, 40, 60, 70, or 240 amino acids). Regions with wavy lines indicate altered frame polypeptide sequences. The numbers above the protein sequences indicate codons.

RESULTS

5′ UTR of *sopE* is not important for SPI-1 TTSS-dependent secretion. Recent reports concerning *Yersinia* have presented evidence that the signal for secretion of some effector proteins resides in the 5′ UTR and coding region of the mRNA (3, 4, 7, 32, 33). To assess the importance of the 5′ UTR in SopE secretion, the *sopE* promoter (P$_{sopE}$) and 5′ UTR were replaced by a heterologous 5′ UTR from the arabinose-inducible promoter P$_{BAD}$ (pMKP$_{BAD}$). Under the control of the arabinose-inducible P$_{BAD}$ promoter, SopE was secreted at levels similar to the levels observed for wild-type *S. enterica* serovar Typhimurium SL1344, as determined by Western immunoblotting (Fig. 2). To further test the role of P$_{sopE}$ and the 5′ UTR in the type III secretion process, we fused these regions to a promoterless β-lactamase gene (pMKP$_{sopE}$) and assessed secretion of β-lactamase (*bla*) via Western immunoblotting with anti-β-lactamase antibodies. P$_{sopE}$ failed to direct secretion of β-lactamase despite the fact that the protein was expressed cytoplasmically (Fig. 2A). β-Lactamase with its native promoter and signal sequence was successfully produced cytoplasmically and secreted in cells containing the control plasmid pBR322, but no cytoplasmic expression or secretion was observed in cells containing the promoterless and signalless plasmid vector pAJR104 (Fig. 2B). This evidence suggests that the 5′ UTR of *sopE* mRNA is not essential for TTSS-mediated secretion of SopE in *Salmonella*.

Amino terminus of SopE mediates SPI-1 TTSS-dependent secretion. Translocation of effector proteins in pathogens such as *Yersinia* is mediated via a protein sequence located at the N

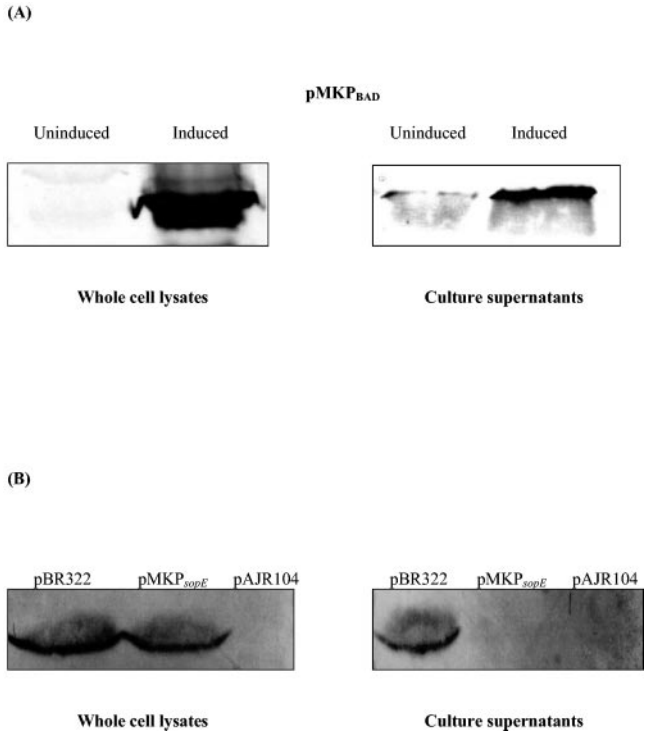

FIG. 2. Secretion of SopE is independent of the 5′ UTR mRNA. A heterologous promoter (P_{BAD}) and 5′ UTR mRNA were fused to *sopE* (pMKP$_{BAD}$), and the full SopE protein was expressed as a Strep-tagged fusion under induced or uninduced conditions (A). Alternatively, the native promoter (P_{sopE}) and 5′ UTR mRNA were fused to a promoterless β-lactamase gene (*bla*) (pMKP$_{sopE}$) (B). Expression was carried out in *S. enterica* serovar Typhimurium SL3261, and whole-cell lysates and culture supernatants were examined for the presence of SopE and for the presence of β-lactamase by Western immunoblotting with antibodies against the Strep tag (A) and β-lactamase (B), respectively, as described in Materials and Methods. Control expression from the promoterless plasmid pAJR104, as well as the signal-containing pBR322, is also shown in panel B.

terminus of the effector protein. A conserved amino acid signal (WEK[I/M]XXFF) has been identified in seven proteins secreted by the type III secretion systems in *Salmonella* (27). For other effector proteins, however, including SopE, there has been no indication of a signaling motif that directs their secretion. In order to delineate the minimal sequence required to direct secretion of SopE, nested deletions of SopE fused to a 9-amino-acid HSV tag that were used to immunologically monitor the fate of the expressed protein were constructed, and their secretion was assessed. Only constructs containing 60 or more amino acids were secreted, suggesting that the signal resides within the first 60 codons of *sopE* (Fig. 3A). The stabilities of the truncated protein products did not change, as determined by using a previously established protocol (11) (Fig. 3B). The stability of the mRNA in constructs consisting of 60 or fewer codons was not affected, as shown by Northern dot blotting and subsequent densitometry (Fig. 3C). Secretion of the 70-amino-acid construct was completely abolished in an SPI-1 null mutant background (SL3262) (Fig. 3A), signifying that TTSS secretion requires a functional SPI-1.

5′ *sopE* mRNA does not play a role in secretion. For *Yersinia* and *Escherichia coli*, there is significant amount of data suggesting that an RNA signal sequence directs secretion of effector proteins (3, 5, 7, 32, 34). So far, there have been no reports of such a system operating in *Salmonella*. In order to clarify the importance of mRNA signals in SopE secretion, frameshift mutations that resulted in a protein with an altered amino acid sequence were engineered. Considering the importance of the amino-terminal region of SopE for secretion, we introduced only the minimal change needed to achieve a frameshift and avoid translation termination codons. Assessment of secretion in constructs expressing altered 70 amino-terminal amino acids from pMKAF70 revealed that the altered frame product was produced cytoplasmically but was not secreted (Fig. 4B). To further investigate the role of the amino-terminal 15 codons of *sopE*, we altered the frame between codons 1 and 15, leaving the chaperone binding site in the region between codons 16 and 70 intact. Alteration of the first 15 amino acids in the presence of the native chaperone binding segment, represented by expression construct pMKAF15, resulted in the protein localizing in the cytoplasm but not being secreted (Fig. 4B). This suggests that mRNA is not likely to be involved in SopE secretion and also demonstrates that the first 15 amino acids have an important role in secretion.

Determination of important regions for SopE secretion. Data presented in this paper provide evidence which suggests that the amino-terminal SopE protein sequence and not the 5′ UTR or the mRNA directs secretion of SopE. We attempted to further investigate the possible localization of such a signal. Alignments of SopE and SopE2, a related *Salmonella* SPI-1 TTSS-secreted effector, by using GeneJockey II revealed the presence of conserved amino acid regions in the chaperone binding domain (Fig. 5). Secretion data obtained during this investigation highlighted the importance of the region between amino acids 40 and 60 for secretion and also for stability (Fig. 3). Further in silico analysis of this amino-terminal segment of SopE by using MOTIF revealed the presence of two putative modification sites (SER [amino acids 50 to 52] and SHK [amino acids 55 to 57]). The presence of such regions in SopE2 as well as in SopE indicated that further investigation was warranted. We changed the amino acid sequence of SopE in the region between codons 50 and 57 using frame-altering mutations introduced by inverse PCR (see Materials and Methods). The absence of these putative motifs in expression products of pMK70Δ resulted in cytoplasmic production of the protein at marginally lower levels than the levels observed for wild-type pMK70 but did not affect its secretion (Fig. 4B). Protein expressed from pMK70Δ exhibited stability similar to that of the native polypeptide produced by pMK70, pMKAF15, or pMKAF70, as determined by Western blotting, suggesting that protein stability was not affected (Fig. 6A). We also compared mRNA levels from the constructs expressing the HSV-tagged products by conducting Northern dot blotting using a probe designed to hybridize with the HSV-tag-encoding mRNA (Fig. 6B). The levels of mRNA expressed from constructs pMK70, pMKAF70, pMKAF15, and pMK70Δ were similar, and there were no significant reductions, as determined by densitometry (Fig. 6B). Collectively, the data suggest that the observed marginal difference in protein levels resulting from alteration of the putative modification sites at codons

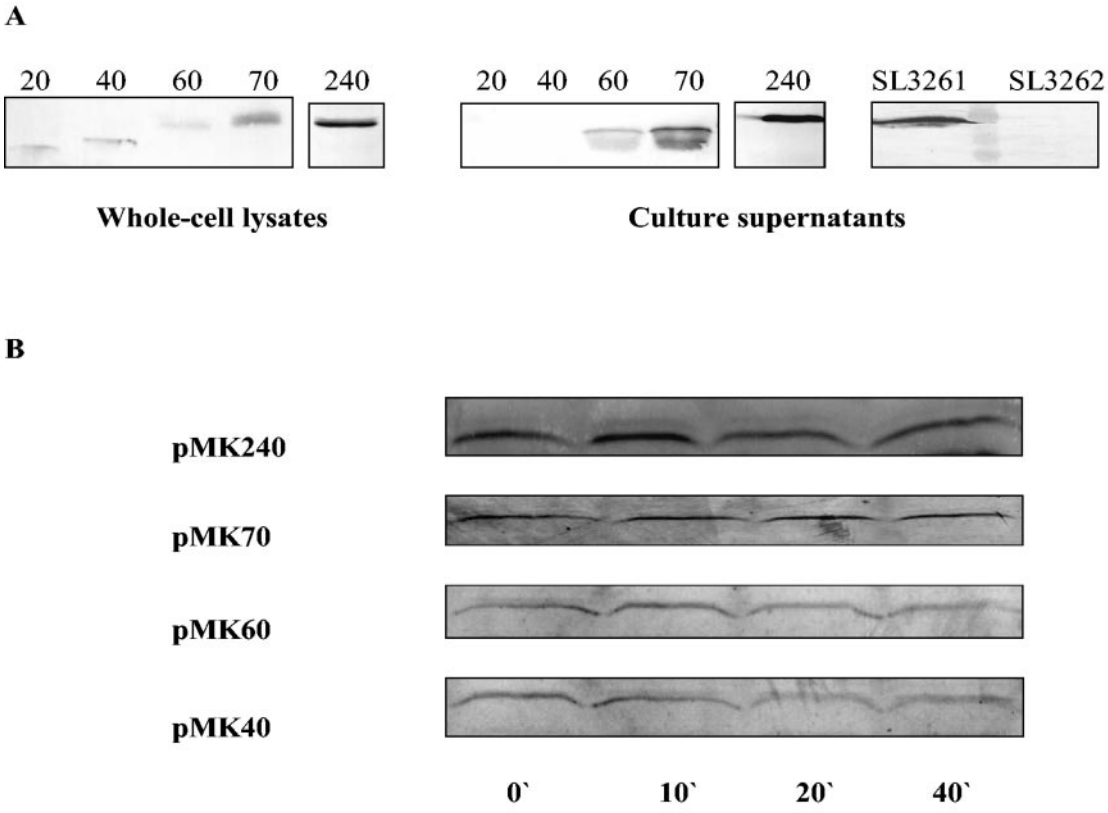

FIG. 3. The first 60 amino acids of SopE are required for the SPI-1 TTSS-dependent secretion of SopE. (A) Western blots demonstrating cytoplasmic expression and secretion patterns of the SopE-HSV tag fusion proteins in *S. enterica* serovar Typhimurium SL3261. Lanes 20, 40, 60, 70, and 240 contained products expressed from constructs pMK20, pMK40, pMK60, pMK70, and pMK240, respectively. Secretion of the 70-amino-acid construct is abolished in the SPI-1-deficient strain SL3262. (B and C) Stability of the protein (B) and mRNA (C) expressed from the constructs. For protein stability, after 4 h of subculturing, 100-μl aliquots were removed (zero time), and chloramphenicol was added to the remaining cultures. Aliquots (100 μl) were removed at 0, 10, 20, and 40 min and separated by SDS-PAGE. Protein levels were analyzed by Western immunoblotting by using an anti-HSV tag. For mRNA stability, after 4 h of subculturing, 1 ml of the culture was processed to isolate total RNA, which was then applied onto a positively charged nylon membrane and probed with digoxigenin-labeled OMW33 (see Materials and Methods).The background represents levels detected from an isogenic strain not expressing the HSV-tagged mRNA. Densitometry was used to obtain relative values for blots. The data are means ± standard deviations of three independent determinations.

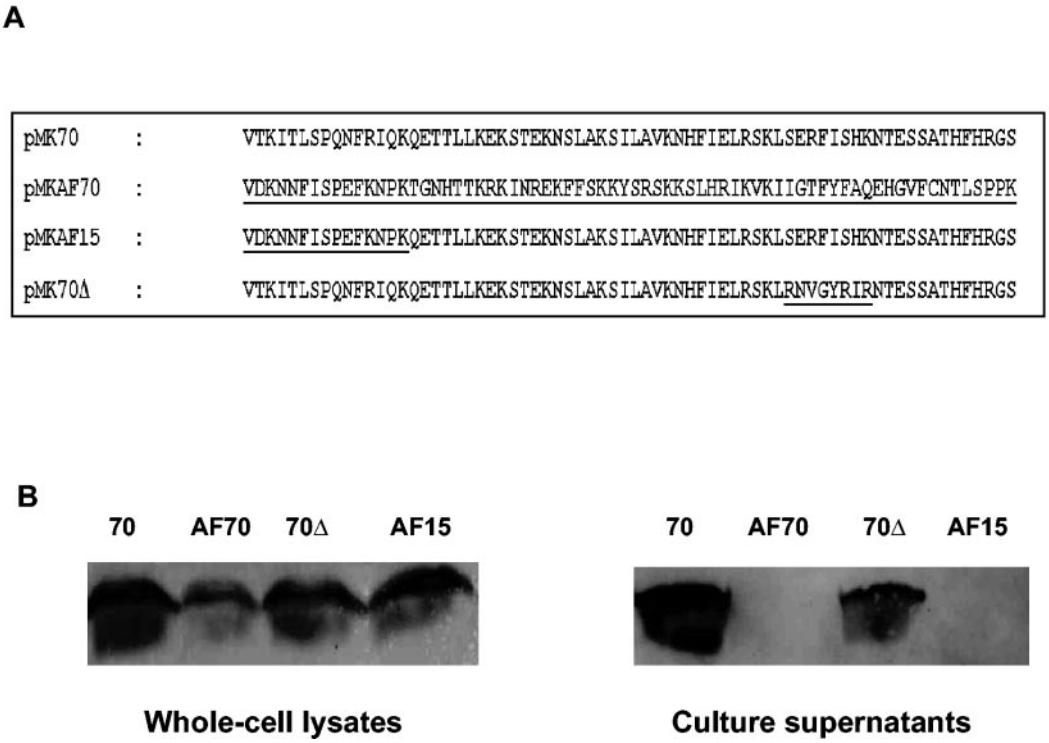

FIG. 4. Introduction of frameshift mutations into the amino-terminal region of SopE affects its secretion through the SPI-1 TTSS. (A) Schematic representation of the expected amino acid sequences of expression products from constructs pMK70, pMKAF70, pMKAF15, and pMK70Δ, with the altered amino acids underlined. (B) Expression of a 70-amino-acid amino-terminal SopE-HSV tag fusion (pMK70 [70]) and various frameshift mutation versions of this fusion (pMKAF70 [AF70], pMK70Δ [70Δ], and pMKAF15 [AF15]) assessed in *S. enterica* serovar Typhimurium SL3261. Whole-cell lysates and culture supernatants were examined for the presence of HSV-tagged proteins by Western immunoblotting by using an antibody directed to the HSV tag as described in Materials and Methods.

50 to 57 is not attributable to either reduced protein or mRNA stability.

DISCUSSION

The signal sequence required for secretion through the TTSS apparatus has been determined for a number of TTSS-secreted proteins (11, 25, 27, 28). In *Yersinia* TTSS secretion is mediated by the amino-terminal mRNA sequence corresponding to the first 15 codons of the secreted effector proteins (3, 5, 31, 33). This work has provided information concerning the nature of the secretion signal of the *Salmonella* effector protein SopE. We aimed at identifying the nature of the signal in *Salmonella* and specifically at assessing the importance of the mRNA sequence in secretion.

We assessed the importance of the 5′ UTR of the *S. enterica* serovar Typhimurium SL3261 secreted effector protein SopE in targeting and export of the protein through the SPI-1 TTSS apparatus. We demonstrated that the 5′ UTR of *sopE* does not play a role in SopE secretion through the TTSS by using a β-lactamase signalless reporter. Furthermore, SopE was secreted from the cytoplasm of cells when it was expressed under the control of the heterologous P_{BAD} promoter and 5′ UTR (Fig. 2).

Despite evidence suggesting the presence of the secretion signal within the first 78 amino-terminal codons of SopE, the

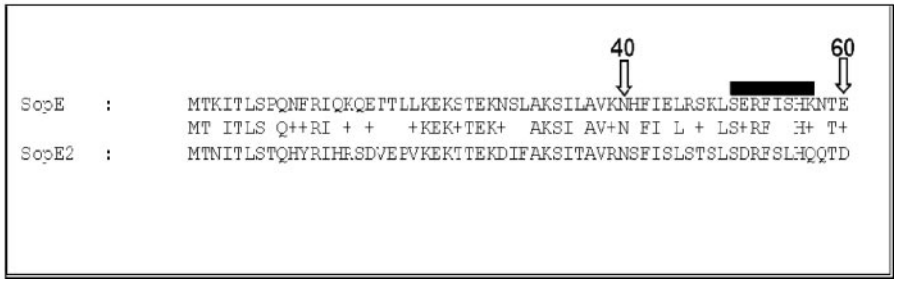

FIG. 5. Comparison of the amino-terminal regions of SopE and SopE2. The amino-terminal regions of SopE and SopE2 were aligned by using GeneJockey II displaying the region of similarity (solid bar) between amino acids 50 and 58.

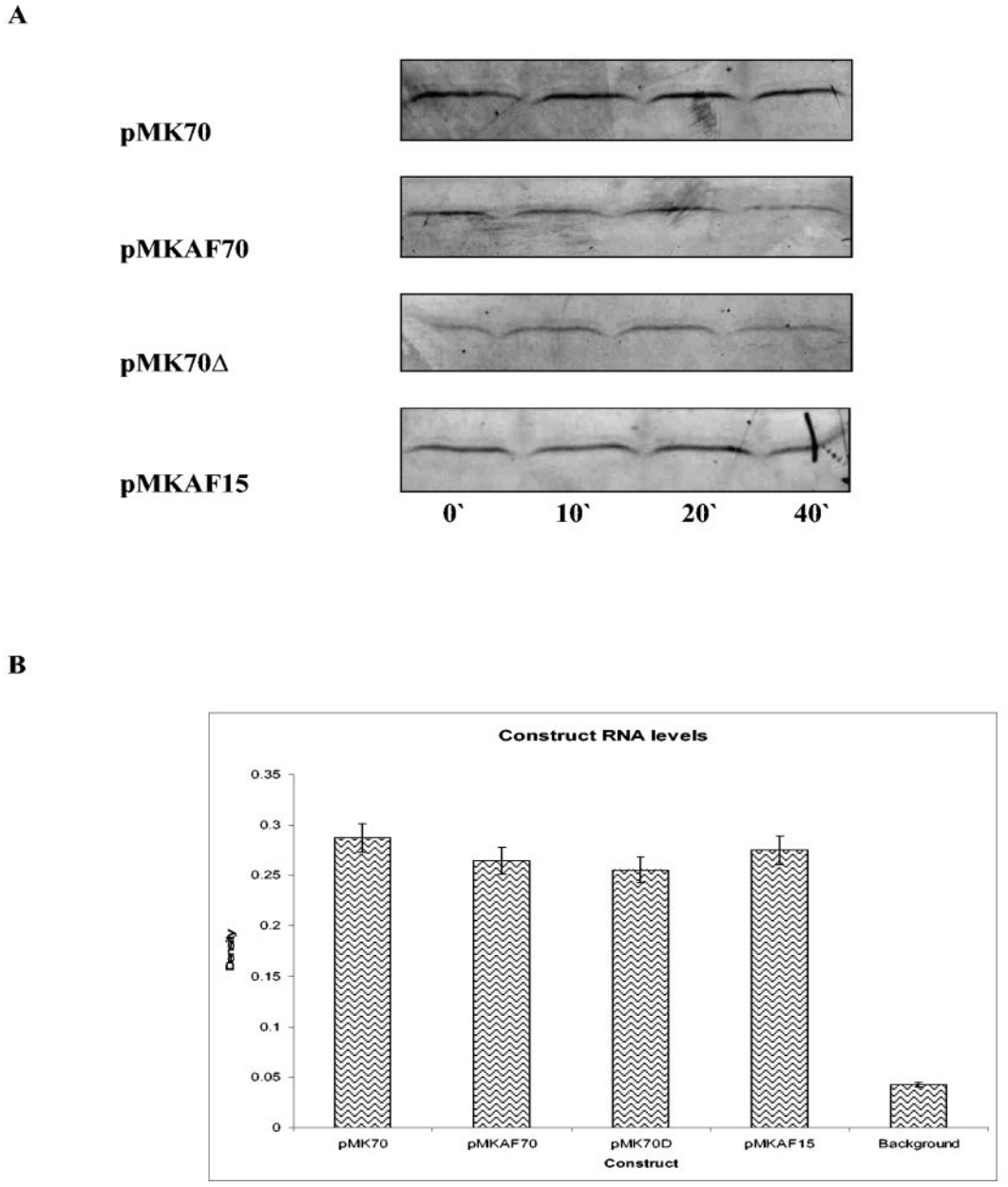

FIG. 6. Frameshift-mediated alteration of the SopE amino-terminal protein sequence does not affect the stability of the protein or *sopE* mRNA. Strains carrying the constructs that were investigated were cultivated under secretion-inducing conditions (see Materials and Methods), and the stability of the expressed protein (A) or mRNA (B) was determined as described in the legend to Fig. 3.

exact nature of this signal was not determined (11, 25). In this work we created a sequential series of amino-terminal fusions of SopE to an HSV reporter epitope. We demonstrated that the first 60 amino acids of SopE is the minimal sequence required for SPI-1 TTSS-dependent secretion (Fig. 3). A 40-amino-acid amino-terminal codon fusion of SopE lacking most of the chaperone binding site was not secreted, highlighting the importance of the region between codons 40 and 60 in SPI-1 TTSS-dependent secretion. Previous data showed that a construct consisting of a fusion between the first 38 amino acids of SopE and PhoA was successfully secreted (25). We repeatedly failed to detect secretion of a 40-amino-acid amino-terminal fusion of SopE with the HSV tag. The stability of the protein and mRNA was measured and was not affected (Fig. 3B and C). To rule out competition for secretion by the native SopE protein, we repeated the study with a *sopE sopE2* isogenic strain and obtained similar results (data not shown). The difference in secretion patterns could therefore be due to a variation in the nature or the size of the fusion tag.

In addition, we assessed the role of the first 15 amino-terminal codons of SopE in the secretion of the protein. In *Yersinia*, TTSS-dependent secretion of effectors is facilitated by the amino-terminal mRNA sequence corresponding to the first 15 codons of the protein (33). In contrast to *Yersinia*, in

Salmonella the mRNA sequence encoded by the first 15 codons of *sopE* is unlikely to play a role in secretion. We were unable to detect secretion from a construct expressing an altered 15 amino-terminal amino acids despite the fact that its 15-codon mRNA sequence was minimally changed by addition of a base (G) and by a substitution (T→A) (Fig. 4B; also see Materials and Methods). This reflects a clear difference in the TTSS secretion mechanisms employed in these two organisms. We also observed that despite the presence of an intact chaperone binding domain, the fusion protein with the altered 15 amino-terminal amino acids was not secreted (Fig. 4B). We therefore suggest that the signal directing secretion of SopE requires the chaperone binding domain, as well as the first 15-amino-acid region.

In an attempt to identify regions of SopE encoding putative motifs directing secretion, we investigated the chaperone binding domain of SopE. Both SopE and SopE2 are secreted via the SPI-1 TTSS, and their amino-terminal regions exhibit a reasonable degree of similarity. Such a region within the SopE chaperone binding domain was identified by using information from protein alignments of SopE and SopE2 (Fig. 5). An in silico motif search identified a putative modification domain containing two putative phosphorylation sites (Fig. 5). The presence of at least one of these sites in both proteins led us to hypothesize that they may be involved in the secretion process. Frame alteration to remove these putative modification sites led to reduced levels of protein expression within the cells but did not affect the levels of secretion (Fig. 4B). The protein stability was unchanged (Fig. 6A), and the levels of mRNA were not altered compared to the control pMK70 levels (Fig. 6B). We were unable to detect any phosphorylation events under a variety of conditions. We are currently conducting further experiments in order to resolve the existence and also the exact nature of possible modifications in the region between amino acids 50 and 57 encoded by *sopE*.

In *Yersinia*, translation of the effector protein YopQ is tightly regulated by YopD, LcrH, YscM1, and YscM2 (2, 7, 8). It has been demonstrated that YscM1 and YscM2 mediate translational control over YopQ by binding to a conserved *yopQ* mRNA sequence, 5′-AUAAA-3′, common to the 5′ UTRs of several effector *yop* genes (7). Surprisingly, we found that although this sequence is absent from the *sopE* 5′ UTR, it is actually a perfect match in the region between codons 50 and 57. Introduction of the frameshift into pMK70Δ resulted in alteration of this conserved regulatory sequence to 5′-CUAU A-3′. However, the marginal reduction in the levels of pMK70Δ is not significant enough to support such a hypothesis. The role, if any, of the conserved *yopQ* mRNA motif in the *sopE* mRNA sequence should be determined in future studies.

In this report we provide data that resolve the location and minimal requirements of important regions that direct secretion of the *Salmonella* effector protein SopE. We obtained data suggesting that the region between amino acids 60 and 70 is not involved in SopE secretion. The role of mRNA in SopE secretion was elucidated and was demonstrated not to be important for the release of the protein through the TTSS apparatus, in contrast to the protein sequence. The importance of the amino acid sequence encoded by the first 15 codons of *sopE* in directing secretion through the SPI-1 TTSS in a manner independent of the chaperone binding site was also highlighted, and its significance as part of the TTSS signal was underlined. Finally, our discovery of a putative conserved *yopQ* binding domain on the mRNA sequence of *sopE* contributes to the ongoing search for conserved sequences involved in the SPI-1 TTSS. Further research is needed to finely map the signal domains in effector proteins in order to fully understand the mechanisms by which these effectors are targeted to the *Salmonella* TTSS.

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